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Circulating lymphocyte populations and autoantibodies in non-obese diabetic (NOD) mice: a longitudinal study

O. PONTESILLI, PATRIZIA CAROTENUTO, L. S. GAZDA, P. F. PRATT & S. J. PROWSE *Barbara Davis Center for Childhood Diabetes, Departments of Pediatrics, Microbiology and Immunology, University of Colorado Health Sciences Center, Denver CO, USA*

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SUMMARY

Several previous observations indicate a role for the immune system in the pathogenesis of insulin-dependent diabetes mellitus (IDDM) in non-obese diabetic (NOD) mice. In order to assess the status of the immune system in this model of spontaneous diabetes we studied the phenotype of circulating lymphocytes and the humoral autoimmunity to islet cells in non-diabetic NOD mice at various ages. Lymphocyte numbers were low in young NOD mice (age < 160 days) as compared with other strains of mice and increased later to reach values in or above the range of controls. The percentages of circulating T lymphocytes and their L3T4$^+$ and Lyt 2$^+$ subsets were higher in NOD mice of all ages and both sexes than in controls; however, no imbalance of the L3T4$^+$ and Lyt2$^+$ subpopulations was found. Anti-insulin autoantibodies were detected by an ELISA assay in all the NOD mice studied throughout the entire period of observation. Autoantibodies reacting with the cytoplasm of islet cells in Bouin's fixed pancreas sections, likely to be anti-insulin antibodies, were found in 47 to 58% of the samples from NOD mice aged 75 to 150 days. Antibodies to surface antigens of rat insulinoma cells were virtually absent in young NOD mice (75–100 days) and appeared in 33 to 43% of the samples from 150 to 185 days old NOD mice. The autoantibodies and the quantitative lymphocyte abnormalities reported here, although not predictive of the appearance of overt diabetes, are likely to be involved in the pathogenesis of the disease and therefore may indicate directions for future investigations.

Keywords NOD mouse insulin dependent (type I) diabetes mellitus lymphocyte subpopulations

INTRODUCTION

The non-obese diabetic (NOD) mouse is a model for human insulin-dependent diabetes mellitus (IDDM) derived from Jcl-ICR cataract mice at Shionogi Research Laboratories, Osaka, Japan (Makino *et al.*, 1980). The appearance of overt diabetes in NOD mice is under the control of sex hormones: castrated females show incidence of diabetes as low as the untreated males, whereas castration increases the incidence of overt disease in males (Makino *et al.*, 1981). More than 90% of NOD mice of both sexes have mononuclear cell infiltration of the islets of Langerhans (Miyazaki *et al.*, 1985) characterized by a predominance of T lymphocytes and presence of B lymphocytes. The pattern of insulitis in NOD mice closely resembles that described in a human diabetic pancreas (Bottazzo *et al.*, 1985). Despite the constant finding of insulitis, the present knowledge of the NOD disease is insufficient to assess the islet destruction mechanism. Cytotoxic activity of NOD spleen

Correspondence: Dr Oscar Pontesilli MD, Barbara Davis Center for Childhood Diabetes, UCHSC Box B-140, 4200 East Ninth Avenue, Denver CO 80262, USA.

cells against semi-allogeneic BALB/c islet cells and anti-islet cell surface antibodies (ICSA) have been reported (Maruyama *et al.*, 1984; Kanazawa *et al.*, 1984) but their relevance to the disease is unknown. However, two recent reports show that the development of diabetes in NOD mice is dependent upon a yet undefined abnormality of the immune system. Ikehara *et al.* (1985) succeeded in transfering insulitis to thymectomized NOD mice by parabiosis and showed that reconstitution of irradiated NOD mice with BALB/c nu/nu bone marrow prevented both insulitis and overt diabetes. Furthermore, overt diabetes can be induced in less than 3 weeks in young non-diabetic NOD mice of both sexes by injection of spleen cells from diabetic NOD mice, provided that the recipient is irradiated before the injection (Wicker, Miller & Mullen, 1986). In order to identify abnormal immunological parameters which may suggest mechanisms of islet destruction and possibly predict the onset of diabetes, we studied peripheral blood lymphocyte populations and circulating autoantibodies in NOD mice at various ages between the development of insulitis and the appearance of diabetes. Anti-islet cell and anti-insulin autoantibodies, and low lymphocyte numbers increasing with age are the major findings reported in this study.

MATERIALS AND METHODS

Mice. NOD/Den mice were bred in our animal facility starting from four pairs kindly provided by Dr Kaichi Kida (Ehime University, Ehime, Japan). In our NOD colony 55% of the females and 22% of the males develop diabetes by 200 days of age. BALB/c, CBA, C3H and DBA/2 mice were obtained from Jackson Laboratory, Bar Harbor, ME.

Blood samples. Peripheral blood was obtained by retroorbital sinus puncture under ether anaesthesia. Plasma for antibody determinations was removed after centrifugation and each sample was reconstituted to the original volume with Hanks' balanced salt solution containing 0·15% sodium azide and 2% fetal calf serum (HBSS-FCS). Blood smears were also prepared and stained with modified Wright-Giemsa staining solution (Stat Stain, VWR cat. n° 48662-172, San Francisco, CA).

White blood cells (WBC), lymphocytes and their subsets. WBC numbers were determined by a Coulter counter model Zf (Coulter Electronics, Hyaleah, FL). Lymphocyte subsets were determined by direct immunofluorescence and cytofluorometry as described (Pontesilli *et al.*, 1986) using the following conjugated antibodies: T24 (anti-Thy 1; Dennert *et al.*, 1980); GK 1.5 (anti-L3T4; Dialynas *et al.*, 1983); 2.43 (anti-Lyt 2·2; Sarmiento, Glasebrook & Fitch, 1980); goat $F(ab')_2$ anti-mouse IgM (Cappel, Cochranville, PA) and rat $F(ab')_2$ anti-mouse IgG (Jackson Immunoresearch, Avondale, PA). Lymphocyte numbers were calculated multiplying the WBC numbers by the percentage of lymphocytes assessed by light scatter properties. In 20 samples the percentage of lymphocytes was determined also by morphology criteria upon examination of blood smears. Since a high correlation ($r = 0{\cdot}969$) was found, lymphocytes have been enumerated only according to their light scatter properties. The majority of the monoclonal antibodies (MoAb) were rat immunoglobulins (Ig), so non-specific binding of rat Ig to mouse lymphocytes was assessed and found less than 1%. Staining of Lyt $2{\cdot}1^+$ lymphocytes in NOD mice was accomplished by indirect immunofluorescence using CL 8921 (Accurate Cedarlane, Westbury, NY) as primary antibody. Percentage of positive cells was determined subtracting the percentage of lymphocytes staining with the second antibody alone.

Insulin autoantibodies (IAA). IAA were detected by a solid phase enzyme linked immunosorbent assay (ELISA) on polystyrene 96-well trays (Dynatech, Immulon II, Alexandria, VA) coated with porcine insulin (Squibb-Novo, Princeton, NJ). Antibody activity was calculated as colog_2 of the serum dilution at which OD_{410} equals the mean + 3 s.d. of 11 background replicates. Sera from NOD mice with IAA activity greater than the mean + 2 s.d. of the activity of 11 sera from BALB/c and C3H mice were scored as positive.

Islet cell antibodies (ICA). Antibodies to cytoplasmic islet cell antigens were detected by indirect immunofluorescence on Bouin's fixed sections of pancreas from BALB/c mice. To test whether the positive staining was due to the presence of anti-insulin antibodies, selected positive samples were

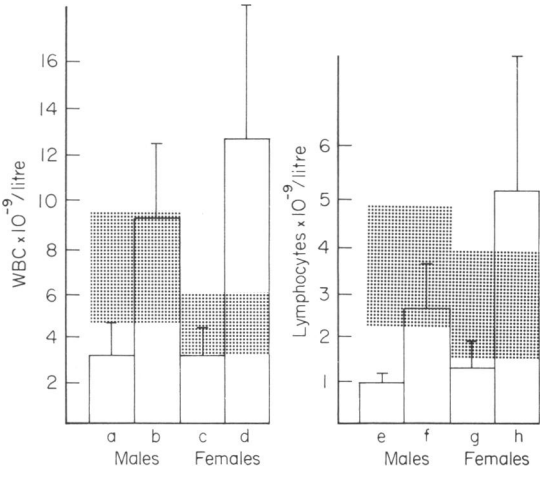

Fig. 1. WBC and lymphocyte numbers in NOD and control mice. Hatched areas represent the mean ± s.d. of DBA/2, BALB/c and CBA controls (age range 60–200 days). Open bars represent the mean of NOD values. NOD groups studied: (a) four males, age range 125–160 days; (b) nine males, age range 175–218 days; (c) 17 females, age range 75–125 days; (d) six females, 175 days old; (e) four males, age range 125–160 days; (f) nine males, age range, 175–218 days; (g) 13 females, age range 102–125 days; (h) six females, 175 days old.

incubated with 1 U porcine insulin (Squibb-Novo, Princeton, NJ) for 30 min at 37°C and then assayed for ICA.

Islet cell surface antibodies (ICA). Antibodies to cytoplasmic islet cell antigens were detected by indirect immunofluorescence on Bouin's fixed sections of pancreas from BALB/c mice. To test whether the positive staining was due to the presence of anti-insulin antibodies, selected positive samples were incubated with 1 U porcine insulin (Squibb-Novo, Princeton, NJ) for 30 min at 37°C and then assayed for ICA.

Immunoglobulin levels. Serum Ig were measured by single radial immunodiffusion (Mancini, Carbonara & Heremans, 1965) in agarose plates purchased from Miles Scientific, Naperville, IL.

Histology. NOD mice were killed at various ages, and pancreases, thyroids, pituitaries, adrenals, stomachs, intestines, livers and spleens were removed, fixed in formalin and sections were stained with haematoxylin–eosin. Pancreas sections were also stained with aldehyde-fuchsin.

Statistics. Mice were grouped by strain, age and sex and compared by one-way analysis of variance; when no significant differences were found, results were pooled and pairs of groups were compared by Student's *t*-test. All statistical evaluations were performed by means of 'Epistat' software (Dr T.L. Gustafson, Round Rock, TX).

RESULTS

White blood cell and lymphocyte numbers. As a first quantitative parameter, WBC were counted in 43 samples from 20 male control mice (age range 62–200 days), 37 samples from 15 female control mice (age range 60–200 days), 13 samples from 11 male NOD mice and 23 samples from 14 NOD females. The majority of the determinations in the NOD group were derived from one litter of eight mice, six females and two males, at 125, 175, 200 and 250 days old. DBA/2, BALB/c and CBA strains were used as controls. No age or strain dependent differences in the number of WBC were found in these control mice. WBC numbers in male controls had a mean ± s.d. of $7058 \pm 2532 \times 10^6$/l, whereas the female controls showed lower WBC numbers ($4401 \pm 1403 \times 10^6$/l, $P < 10^{-6}$ vs male controls). These values are represented as hatched areas in Fig. 1. On the contrary, as shown in Fig. 1, WBC numbers in NOD mice vary with age. Young NOD mice (< 160 days) of both sexes had lower WBC numbers than controls ($3445 \pm 836 \times 10^6$/l in males, $P = 7{\cdot}2 \times 10^{-3}$ vs. control males;

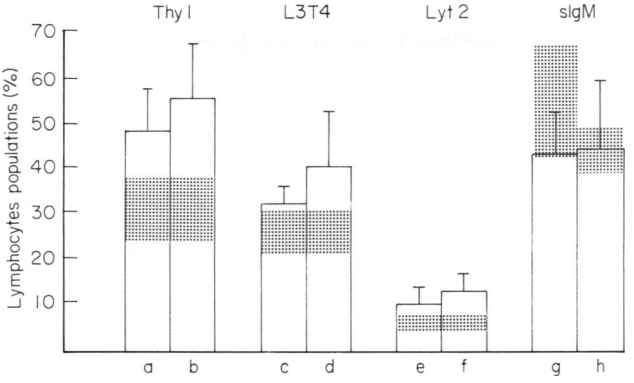

Fig. 2. Percentages of peripheral blood lymphocyte populations in NOD and control mice. Hatched areas represent the mean ± s.d. of control groups composed as follows: Thy 1:38 BALB/c males and females, CBA males and females, and DBA/2 females, age range 62–200 days; L3T4: 11 BALB/c males, age range 100–200 days; Lyt 2: 11 BALB/c males, age range 100–200 days; sIgM: 32 BALB/c, CBA and DBA/2 males, age range 62–200 days; 14 BALB/c and DBA/2 females, age range 96–200 days. Open bars represent the mean of NOD values. NOD groups studied: (a) 15 males, age range 125–218 days; (b) 25 females, age range 102–190 days; (c) 13 males, age range 125–200 days; (d) 18 females, age range 125–190 days; (e) six males, age range 175–200 days; (f) six females, age range 175–200 days; (g) 14 males, age range 125–218 days; (h) 23 females, age range 102–190 days.

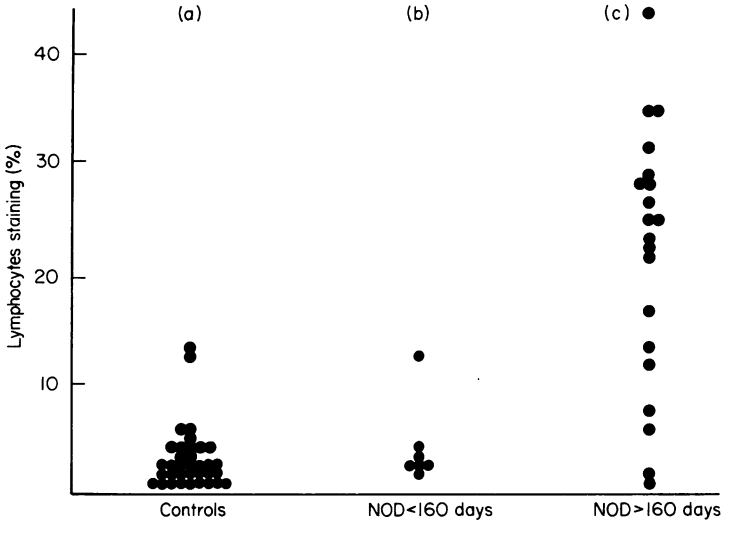

Fig. 3. Percentage of peripheral blood lymphocytes staining with anti-IgG antiserum in NOD and control mice. The groups are as follows: (a) 37 BALB/c, DBA/2 and CBA males and females, age range 60–200 days; (b) seven NOD females, age range 102–106 days; (c) 20 NOD males and females, age range 175–218 days.

$3574 \pm 1158 \times 10^6$/l in females, $P = 3{\cdot}9 \times 10^{-2}$ vs control females). These values were significantly higher in NOD mice older than 160 days when compared with the younger animals of the same strain ($9357 \pm 3436 \times 10^6$/l in males, $P = 6{\cdot}8 \times 10^{-3}$ vs young NOD males; $12667 \pm 5687 \times 10^6$/l in females, $P = 2{\cdot}0 \times 10^{-6}$ vs young females) as well as with controls of the same sex ($P = 2{\cdot}4 \times 10^{-2}$ in males; $P < 10^{-6}$ in females). We chose the age of 160 day to divide the two NOD groups (young vs. old) because that is the average age of diabetes onset in our colony.

Lymphocyte numbers showed a similar behaviour (Fig. 1). Young NOD mice had low numbers of circulating lymphocytes when compared with controls of the same sex ($963 \pm 83 \times 10^6$/l vs.

Table 1. Incidence of autoantibodies (positive samples/samples tested) and Ig levels in NOD mice

Age (days)	75	100	125	150	185	200	225	250
ICA								
M	3/5	4/6	1/6	5/9	1/6	4/7	2/5	1/7
F	1/3	3/6	6/8	3/8	2/6	0/3	0/1	0/6
Total	4/8	7/12	7/14	8/17	3/12	4/10	2/6	1/13
IAA								
M	4/4	3/4	4/5	4/4	3/5	—	3/5	5/5
F	1/1	2/2	3/3	3/3	3/3	—	0/1	1/1
Total	5/5	5/6	7/8	7/7	6/8	—	3/6	6/6
ICSA								
M	0/5	0/6	1/6	2/9	4/5	1/7	—	1/2
F	0/5	1/9	3/9	4/9	3/11	3/7	—	2/5
Total	0/10	1/15	4/15	6/18	7/16	4/14	—	3/7

	Ig levels (mg/dl)		
Age (days)	IgG	IgA	IgM
75	460 ± 44* (7)	146 ± 30 (7)	11·0 ± 2·3 (7)
100	511 ± 50 (7)	167 ± 28 (7)	12·4 ± 2·3 (7)
125	583 ± 85 (8)	230 ± 35 (8)	15·6 ± 3·3 (8)
150	569 ± 128 (8)	299 ± 15 (8)	16·9 ± 3·1 (8)
C3H males (70 day old)	495 ± 87 (8)	121 ± 32 (8)	17·9 ± 3·0 (8)

M, males; F, females.
* Mean ± s.d.

$3495 \pm 1446 \times 10^6$/l, $P = 1{\cdot}3 \times 10^{-3}$ in males; $1300 \pm 544 \times 10^6$/l vs $2686 \pm 1229 \times 10^6$/l, $P = 4{\cdot}4 \times 10^{-4}$ in females). Lymphocytes increased significantly in the older NOD groups ($2620 \pm 1026 \times 10^1$/l in males, $P = 9{\cdot}3 \times 10^{-3}$ vs young NOD males; $5366 \pm 3021 \times 10^6$/l in females, $P = 2{\cdot}4 \times 10^{-2}$ vs young NOD females). In contrast to what was found for the WBC numbers, only the older NOD females showed higher lymphocyte numbers than sex matched controls ($P = 1{\cdot}3 \times 10^{-3}$) whereas the values of the older NOD males were in the range of controls.

It has been recently reported that lymphopenic BB rats undergoing frequent blood withdrawal (15–27% of the total blood volume each time) showed an increase of lymphocyte numbers accompanied by normalization of lymphocyte subsets and lower incidence of diabetes (Yale, Grose & Marliss, 1986). Since a relevant number of NOD mice underwent repeated bleedings, we considered the possibility that the observed increase with age of WBC and lymphocyte numbers was caused by this procedure. Blood was drawn 3–4 times from CBA and DBA/2 mice at an average interval of 35 days between two consecutive bleedings. No significant age dependent increase of WBC and lymphocyte numbers was found in these control mice (data not shown). We therefore conclude that the rise observed in NOD mice was not induced by the repeated bleedings.

Circulating lymphocyte populations. To characterize the peripheral blood lymphocytes in NOD mice, we determined the percentage of T and B lymphocytes, defined as Thy 1^+ and surface IgM^+ ($sIgM^+$) cells respectively. In a smaller group of NOD mice class I and class II reactive T cells, defined as $L3T4^+$ and $Lyt2^+$ cells respectively, were analysed. DBA/2, BALB/c and CBA mice were used as controls for these parameters. No age or sex dependent differences were found in the percentages of lymphocyte populations both in NOD and control mice, with the exception of the percentage of $sIgM^+$ lymphocytes which was lower in female controls than in male controls. As

shown in Fig. 2, NOD mice had higher percentages of circulating Thy 1^+ lymphocytes than controls ($52{\cdot}1 \pm 11{\cdot}7\%$ vs $33{\cdot}5 \pm 10{\cdot}2\%$, $P < 10^{-6}$) and this difference is reflected in the percentages of L3T4$^+$ cells ($32{\cdot}4 \pm 3{\cdot}7\%$ in NOD males and $40{\cdot}0 \pm 12{\cdot}2\%$ in NOD females vs $25{\cdot}8 \pm 5{\cdot}0\%$ in controls, $P = 1{\cdot}3 \times 10^{-4}$ and $P = 9{\cdot}9 \times 10^{-4}$ respectively) and Lyt 2^+ cells ($11{\cdot}4 \pm 4{\cdot}0\%$ in NOD mice vs $6{\cdot}7 \pm 1{\cdot}5\%$ in controls, $P = 1{\cdot}6 \times 10^{-3}$). The ratios L3T4$^+$/Lyt 2^+ in 12 NOD mice ($3{\cdot}5 \pm 1{\cdot}7$) and 11 BALB/c mice ($3{\cdot}9 \pm 0{\cdot}8$) were not significantly different. The percentage of sIgM$^+$ cells in NOD females was in the range of controls ($43{\cdot}4 \pm 15{\cdot}0\%$ and $45{\cdot}2 \pm 5{\cdot}0\%$ respectively), whereas control males showed values ($54{\cdot}1 \pm 12{\cdot}2\%$) higher than both the NOD male ($42{\cdot}0 \pm 9{\cdot}1\%$, $P = 1{\cdot}7 \times 10^{-3}$) and the control female groups ($45{\cdot}2 \pm 5{\cdot}0$, $P = 1{\cdot}4 \times 10^{-2}$). Lymphocytes staining with anti-IgG antiserum (sIgG$^+$) were present in a very low percentage in 37 samples from control mice and in seven young NOD females ($3{\cdot}4 \pm 2{\cdot}7\%$ and $4{\cdot}5 \pm 3{\cdot}5\%$ respectively) but, as shown in Fig. 3, they have been found in significantly higher percentages in older NOD mice ($22{\cdot}0 \pm 11{\cdot}3\%$; $P < 10^{-6}$ vs controls).

Some of the data reported above were derived from a litter of eight NOD mice studied longitudinally; three of the six females and none of the males became diabetic before 250 days of age. Their lymphocyte numbers and populations reflected what has been outlined for the whole group of NOD mice and none of these parameters was predictive of the subsequent appearance of diabetes.

Histology. Organs for histology evaluation were obtained from 18 NOD mice at 100 days, 160 days, 200 days and 250 days of age. Mononuclear cell infiltration of the pancreatic islets was present in all pancreases but one, and ranged from mild insulitis (presence of mononuclear cells at one pole of some islets with no or minimal tissue disruption and/or degranulation) to complete substitution of most islets with infiltrate or fibrous tissue in all age groups. High variability of the degree of insulitis was seen also in each single pancreas, the detection of both intact and heavily infiltrated islets in the same section being common. A moderate to heavy perifollicular mononuclear cell infiltration was found in three of the 17 thyroids examined, with little or no alteration of the follicle architecture. No abnormalities were found in spleens, pituitaries, livers, intestines, stomachs and adrenals of all the animals studied.

Autoantibodies. Islet cell and anti-insulin autoantibodies were detected in several serum samples from NOD mice at various ages and the results are summarized in Table 1. IAA were found in virtually all NOD sera studied at all ages with an activity of $11{\cdot}4 \pm 2{\cdot}0$ (specific enzymatic conversion of the ABTS substrate was present on average for dilutions lower than 1:2702), significantly higher than the activity of control sera ($P < 10^{-6}$). Ten out of 13 control sera from BALB/c and C3H mice had IAA activity $< 3{\cdot}3$ (i.e. negative at a 1:10 dilution); the remaining three control sera tested had activities of 9·5, 9·8 and 12·1 respectively. All NOD samples for IAA determination were obtained from one single litter of five males and three females. Of these animals only one female developed diabetes before 250 days of age. Serum Ig levels were also determined in this litter at various ages and are reported in Table 1 together with the Ig levels of a group of 70 day old male C3H mice. A significant increase with age of all three Ig classes was observed in NOD mice ($P = 0{\cdot}041$ for IgG, $P < 10^{-6}$ for IgA, $P = 0{\cdot}001$ for IgM).

The staining pattern of ICA positive sera on mouse Bouin's fixed pancreas section is shown in Fig. 4a. Eight control sera from BALB/c and C3H mice were all negative for ICA whereas 47 to 58% of the sera from NOD mice aged between 75 and 150 days were positive. The incidence of ICA decreased to less than 40% in the older NOD groups. Two samples showing strong reactivity in the ICA assay were tested after preincubation with porcine insulin. The positive staining of both samples was totally abolished as shown in Fig. 4b. No other positive samples were tested after preincubation with insulin.

ICSA were virtually absent in young NOD mice (75–100 days) and peaked between 150 and 185 days of age (33 to 43% of the sera were positive). However, no significant difference between NOD mice and controls was found in the percentage of specific chromium release (ICSA were found in nine out of 22 control sera from BALB/c and CBA mice) and this strongly limits the value of this assay in mice. Cytotoxic activity of positive sera was not abolished by absorption with rat liver powder, so we considered it specific for islet or RIN5F cell antigens.

All autoantibodies were found both in female and male NOD mice regardless of subsequent onset of diabetes.

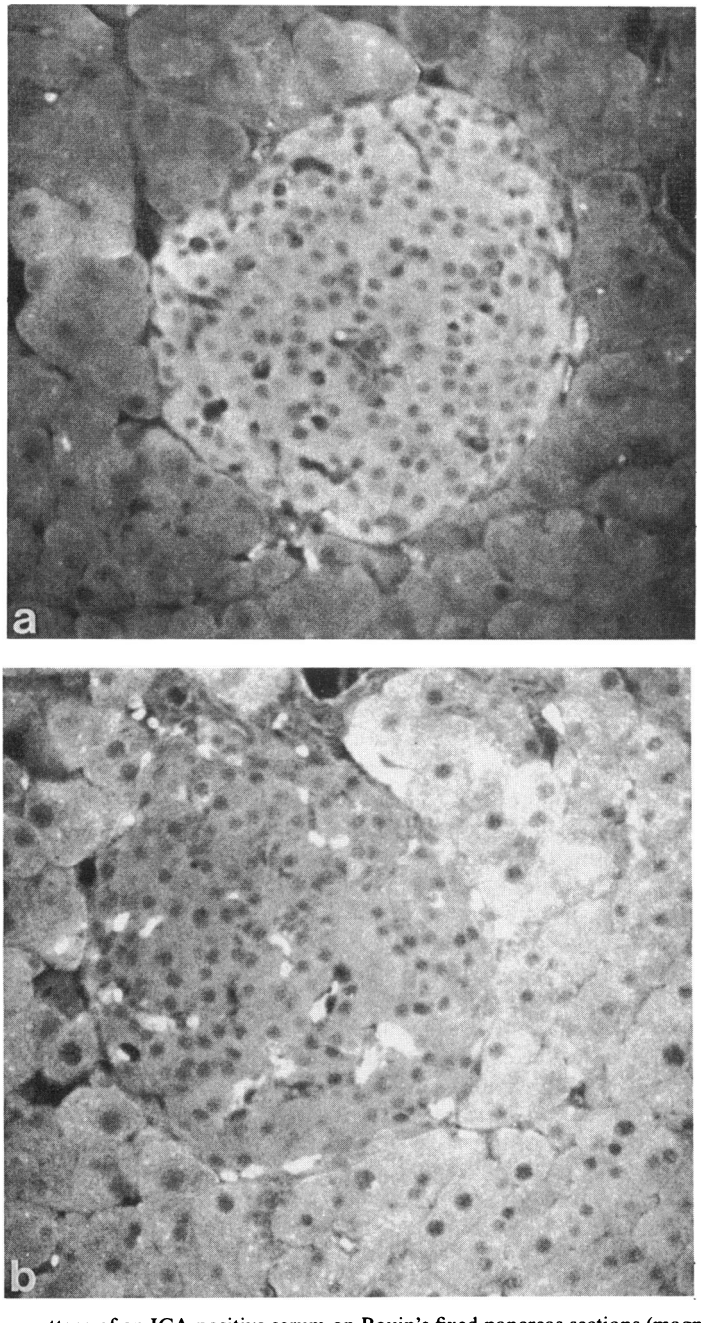

Fig. 4.(a) Staining pattern of an ICA positive serum on Bouin's fixed pancreas sections (magnification × 250). (b) Binding to Bouin's fixed pancreas section is abolished by preincubation of an ICA positive serum with insulin.

DISCUSSION

The constant presence of mononuclear cell infiltration in the islet of Langerhans of NOD mice (Miyazaki *et al.*, 1985) and the recent finding that diabetes can be rapidly induced in irradiated non-

diabetic young NOD mice by injecting spleen cells from diabetic NOD mice (Wicker, Miller & Mullen, 1986) strongly support the hypothesis of a participation of the immune system in the disease process. However, the presence of insulitis is not sufficient for the appearance of diabetes in these animals. The histological evaluation reported in the present study clearly shows that all degrees of mononuclear cell infiltration and destruction of pancreatic islets are virtually always present at all ages and in both sexes, whereas the incidence of overt diabetes is relatively lower, especially in males. Sex hormones have been suggested to be a crucial factor in the development of overt disease (Makino *et al.*, 1981) but other factors may be participating in the process. Furthermore, mononuclear cell infiltration of other organs, namely thyroids (this study) and salivary glands (M. Hattori, pers. comm.) is found in some animals with no known associated functional abnormality.

In the attempt to provide useful markers of disease and indicate possible mechanisms of islet cell destruction we studied the peripheral blood lymphocyte phenotype and the presence of autoantibodies in NOD mice focusing our attention on the time between the development of insulitis and the appearance of overt diabetes. We found that lymphocyte numbers in NOD mice younger than 160 days were significantly lower than control values, but older NOD mice showed lymphocyte numbers in the range of controls, or even higher in females. The percentage of circulating T lymphocytes in NOD mice was always higher than in controls, similar to the percentage of splenic T lymphocytes reported in 3 to 13 week (20–90 day) old NOD mice by Miyazaki *et al.* (1985). Low absolute numbers of T lymphocytes were reported both in the peripheral blood and in the spleen by Kataoka *et al.* (1983) in 12 week old NOD females compared with age matched ICR mice. We extend this observation showing that older NOD mice have circulating lymphocyte numbers comparable to or higher than controls. We can therefore conclude that the previously reported T lymphopenia is not a constant feature of NOD mice and is not an absolute requirement for the development of overt diabetes, as proposed for the BB rat (Guttman *et al.*, 1983). The lower number of lymphocytes at younger ages may well be a reflection of immature immunoregulatory mechanisms possibly related to the development of insulitis. However no imbalance of the major T-lymphocyte subsets, as defined by their phenotypic markers, was found in this study. We exclude the possibility that the rise in lymphocyte numbers observed in the present study is due to the bleeding regimen, since control mice undergoing the same treatment did not show significant variations of this parameter. The percentage of circulating B cells, defined as $sIgM^+$ cells, had high variability both in NOD and control mice and, on the whole, no significant differences in the groups studied were found. Interestingly, the older NOD groups showed high percentages of $sIgG^+$ lymphocytes, virtually absent in the younger NOD mice and in controls. This finding may be due to IgG absorbed onto the Fc receptor and complement receptor bearing lymphocytes and strongly suggests the presence of circulating immune complexes (Paraskevas *et al.*, 1972; Eden, Bianco & Nussenzweig, 1973).

Human type I diabetes is characterized by the presence of several autoantibodies, namely ICA detected on frozen sections of human type O pancreas (Bottazzo, Florin-Christensen & Doniach 1974), ICSA (Lernmark *et al.*, 1978) and IAA (Palmer *et al.*, 1983). We found similar autoantibodies in the NOD mouse; however, the characteristics of ICA seem to be different from those of their human counterparts. NOD sera were negative when tested on frozen sections of mouse pancreas in a preliminary screening (S. J. Prowse, unpublished observation). We have been able to detect ICA in NOD sera using Bouin's fixed pancreas as substrate. Two major differences between frozen and Bouin's fixed tissue are: (a) the absence in the latter of glycolipids which have been indicated as a possible target of human ICA (Nayak *et al.*, 1985) and (b) a low insulin content in frozen sections which makes Bouin's fixed pancreas a more suitable substrate for detection of anti-insulin antibodies (Yagihashi *et al.*, 1982). Preincubation of two ICA positive sera with insulin resulted indeed in the abolishment of their reactivity on Bouin's fixed sections; this strongly suggests that the assays used for ICA and IAA in this study are detecting antibodies of the same specificity, with greater sensitivity in the IAA ELISA assay. IAA were present since the beginning of the study but in a much higher proportion of animals than the other autoantibodies studied. Antibodies to insulin are likely to be responsible for an altered glucose homeostasis, either directly, binding insulin molecules, or via anti-idiotype antibodies which may block the insulin receptors (Elias *et al.*, 1984).

The incidence of ICSA in our study is similar to that reported by Kanazawa *et al.* (1984); ICSA appear at an age when insulitis is known to be already developed (> 100 day) and this suggests that these antibodies do not play a role in the initial islet damage. The detection of ICSA in a considerable number of our control mice strongly limits the value of this finding and questions the validity of this assay in mice. Further studies on sera and monoclonal antibodies from NOD mice are now in progress in our laboratory in order to define the biochemical specificity and the possible meanings in disease of the autoantibodies described here.

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